The Application of Variable Chlorophyll Fluorescence to Microphytobenthic Biofilms

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Abstract

The successful application of variable chlorophyll fluorescence methodology to higher plants and other phototrophs inspired workers in the 1990s to apply the methods to microalgal communities inhabiting benthic soft sediments, the microphytobenthos (MPB) of estuarine and other coastal habitats. It was quickly identified that particular aspects of the physiology (cellular vertical migration within the sediment matrix), photophysiology (high capacity for down regulation, e.g. NPQ, and chlororespiration in the dark) and the effects of the physical structure of the sediment/biofilm matrix (light attenuation by the matrix itself) confounded the interpretation of fluorescence information obtained. In this chapter, the authors attempt to explain these and other issues pertinent to MPB biofilms and to summarise how methods have been developed to alleviate the problems encountered. Although much work is still needed to fully understand fluorescence data for the MPB, studies to date have been highly illuminating with regard to rhythms of productivity, photoacclimatory mechanisms and the behavioural ecology and physiology of MPB at an integrated biofilm level and at a cellular level. This chapter therefore introduces benthic biofilms and relevant specific fluorescence methodological issues, expands on subsurface fluorescence signal and migration, discusses down regulatory non-photochemical quenching (NPQ) resulting from xanthophylls cycle induction, compares measurement of electron transport rate proxies, examines light curve methodology, and concludes by comparing fluorescence productivity measurements with those of other methodologies such as oxygen evolution and carbon uptake.

Contents

1. Introduction to benthic biofilms

Community assemblages of diatoms, green algae and cyanobacteria comprise the microphytobenthos (MPB) inhabiting benthic sediment ecosystems (Admiraal 1984, Underwood and Kromkamp 1999, Consalvey et al. 2004). Particular attention has been paid top the analysis of intertidal soft sediment systems, e.g. cohesive mudflat and sandy substrata typical of estuarine habitats. Variable chlorophyll fluorescence has been applied to these systems since the 1990s, in an attempt to investigate the primary productivity and photophysiology of the integrated biofilms, when viewed as a "black box system", and also the species level (Sections 5, 6 and 7). These transient biofilms are not confined to such soft sediment habitats however, and more recently application of fluorescence methodologies has been applied to biofilms inhabiting rocky shores and stromatolite systems (Kromkamp et al. 2007; Perkins et al. 2007). However the large majority of published work has centred upon benthic soft-sediment biofilms, due to their important ecosystem functions of carbon flow and sediment stability (Underwood and Kromkamp, 1999). In the former their high magnitude of productivity fuels carbon flow through invertebrate and bacterial food webs to support important trophic levels of anthropogenically exploited taxa, including coastal fish and shell fisheries and coastal avifauna. In the case of sediment stability, biogenic exopolymers, usually referred to as extracellular polymeric substances (EPS), produced by the MPB may contribute significantly to sediment stability, hence increasing the sediment resistance to hydrodynamic stresses and thus resistance to coastal erosion (e.g. Underwood and Kromkamp 1999 and citations there-in).

One attribute of MPB physiology has in particular contributed to confounding the application of fluorescence methodology to benthic biofilms. This is the behavioural adaptation of the MPB to migrate vertically in their sediment matrix habitat in response to environmental stimuli, as well as part of endogenous tidal and diel rhythms (Sections 2, 3, 4, 5 and 6). Consalvey et al. (2004) reviewed this migration in MPB biofilms and Consalvey et al. (2005) discussed the effect of this as part of a review of fluorescence methodology applied to MPB biofilms. Vertical migration appears to follow tidal and diel rhythms such that cells migrate to the surface of the sediment to coincide with daylight emersion periods, whilst migrating back in to the sediment for immersion periods (Consalvey et al. 2004 and citations there-in). In some instances this appears to be modulated by light environment such that low turbidity in the overlaying water column enables the MPB to remain at the sediment surface after the onset of immersion, and during bright moonlit emersion periods, MPB will migrate to the surface (authors pers. obs.).

Light is undoubtedly a major stimulus in MPB vertical migration. Several studies have shown that cells within MPB biofilms show negative or positive phototaxis. For example, cells will migrate down to avoid potentially harmful high light environments and migrate up to optimise the light environment when ambient light levels are low (Sections 2, 3 and 6). Cells also show “microcycling” such that their can be a constant turnover of taxa at the sediment surface, such that cells shade each other resulting in a reduction in photodose integrated over time (Section 2, 5,, 6 and 7). As well as affecting productivity, such “behavioural” down regulation of photosynthesis may act to make interpretation of fluorescence measurements difficult. For example, cells will migrate to different positions within the sediment matrix making the distance between the cells and the fluorometer probe variable and

unknown. This leads to a variable attenuation of applied actinic light, as well as the fluorescence yields used to study aspects of photoacclimation. Furthermore, cells may migrate in response to darkness applied for measurement of dark adapted fluorescence parameters, again altering fluorescence yields (Sections 2, 3 and 6). These effects, and others discussed in this chapter, lead to potential errors in the application of variable chlorophyll fluorescence to migratory MPB biofilms. As a result great care is needed in the interpretation of fluorescence data obtained. Benthic diatom taxa, which can comprise the majority of MPB biomass, also show differences in photophysiology from higher plants, making conventional interpretation of fluorescence data incorrect. For example, diatoms exhibit high levels of down regulation through diadinoxanthin/diatoxanthin xanthophyll cycling, non-photochemical quenching (NPQ), induced as a result of the trans-thylakoid proton gradient resulting from light induced electron transport (Section 3). Thus MPB cells can show behavioural and physiological down regulation. However chlororespiration during dark periods leads to retention of this proton gradient, retaining NPQ in the dark (Section 3). This can suppress maximum fluorescence yield in the dark (Fm) such that it is lower than operational maximum yield in low light (Fm'), making conventional calculation of NPQ from the difference in these yields problematic (Sections 3 and 5).

In this review, the authors have summarised the main areas of fluorescence research as applied to benthic biofilms, principally concentrating on those of intertidal soft sediment ecosystems. The methodology is summarised, along with potential problems, as well as ways to minimise error and achieve correct interpretation of data obtained. The review covers subsurface signal as a result of vertical integration of fluorescence measurements (Section 2), non-photochemical quenching and the xanthophyll cycle (Section 3), measurement and calculation of fluorescence derived

electron transport rate (Section 5), methodology used to obtain light curve parameters of photophysiology, e.g. photoacclimation (Section 6) and concludes with a section comparing fluorescence to other methods including radio-labelled carbon uptake and oxymetry (Section 7).

2. The effects of subsurface signal

2.1 Microphytobenthic biofilms on soft sediments

Sediments colonised by microphytobenthos are optically dense, causing both downwelling light (including light generated by a fluorometer) as well as upwelling fluorescence to suffer from considerable attenuation within the microalgal biofilm/sediment matrix. As a result, the fluorescence levels F_s and F_m' measured near the surface represent the integration of the fluorescence signals emitted at various depths, distorting the relationship between F_o and chl a as this relationship will thus not only depend upon the total biomass, but also on the shape of the vertical biomass profile. Of more importance, this effect causes the measurement of the effective quantum yield, $\Delta F/F_m$', at the surface to differ significantly from the true, inherent value of the microalgae composing the biofilm, because the relationship between F_s and F_m' varies with irradiance within the depth range over which the fluorometer signal is integrated (Forster and Kromkamp 2004, Serôdio 2004).

The depth-integration effect was first predicted from observations of changes in $\Delta F/F_m$' in undisturbed biofilms under constant irradiance (Underwood et al. 1999, Perkins et al. 2001, 2002), and was studied in detail using numerical simulation models (Forster and Kromkamp 2004, Serôdio 2004). Studies showed that fluorescence measurements taken non-invasively at the surface of the sediment result in a substantial light-dependent overestimation of the inherent value of $\Delta F/F_m$' and

relative electron transport rate, rETR, particularly important under supersaturating irradiances (Fig. 2.1). As a consequence, light curves derived from depth-integrated measurements are likely to appear to saturate at higher irradiances, or to be less photoinhibited when compared to the true physiological response of the biofilm-forming microalgae. The contribution of sub-surface fluorescence signals also affect the determination of the light response of non-photochemical coefficient NPQ, which is expected to be underestimated under high light when measured non-invasively in intact biofilms (Forster and Kromkamp 2004, Serôdio 2004).

While this effect may occur, although to a lesser extent, in other optically dense samples such as thick leaves or macroalgal thalli (Forster and Kromkamp 2004, Serôdio 2004, Susila et al. 2004), in the case of microphytobenthos the problem is further complicated by the occurrence of comparatively large scale vertical migration by motile diatoms. In this case, it becomes highly difficult to interpret fluorescence yields from biofilms where the cells move vertically within the sediment matrix, since the subsurface signal emanates not only from cells at unknown depth, but at a variable depth in the sediment. Additionally, signal strength is proportional to the distance between the fluorometer probe and the cells themselves, thus vertical migration may increase or decrease measured yields, so making interpretation of changes in yield or calculation of photophysiological parameters difficult. For example, a decrease in both F_m' and F can be due to induction of NPQ down regulation on exposure to increasing PAR or to downward migration (negative phototaxis). It is often not possible to differentiate between the two processes using fluorescence methods. For this reason it can be prudent to use subsurface spectral reflectance for measurements such as biomass, see below, (Kromkamp et al. 2006, Morris et al. 2008) as the reflectance spectra are not influenced by NPQ.

It is hard to avoid artefacts of tidally induced vertical migration and positive or negative phototaxis but the effects can be minimized by not taking measurements during the first and last hour of the emersion period when vertical migration is maximal. In addition it is better to minimize the effects of phototaxis by keeping the duration of measurements as short as possible (e.g. minimise the duration of light steps during rapid light curves, RLCs, see later). PSII quantum efficiency often stabilizes before true steady state fluorescence is reached (authors pers. obs).

The issue of the subsurface signal from the cells within the subsurface sediment has been investigated in considerable depth (Kromkamp et al. 1998, Perkins et al. 2002, Forster and Kromkamp 2004, Serôdio 2004, Jesus et al. 2006). Kromkamp et al. (1998) discussed the issue of microcycling of cells such that the fluorescence yield was obtained from a varied surface community over time and that this could explain the persistent high $\Delta F/F_m'$ at high incident irradiance. Perkins et al. (2002) reported over estimation of ETR in sediments due to subsurface signal from cells exposed to a lower light level than that applied at the surface and showed that the community composition could change during a light curve. In addition it appeared that the PSII signal was contaminated by PSI-fluorescence, shown by using a 680 nm bandpass filter compared to the more conventional 695 nm longpass filter. Underwood et al. (2005) used high resolution fluorescence imaging to report diel patterns in $\Delta F/F_m'$ of several benthic diatom species which showed microcycling over an artificially extended emersion period (Fig. 2.2). Serôdio (2004) and Forster and Kromkamp (2004) also demonstrated similar effects on measurements due to integration of the fluorescence signal over sediment depth. To make matters more complex, the degree of overestimation of $\Delta F/F_m'$, which manifests the greatest at light levels above the light saturation coefficient, E_k, depends also on the shape of the

biomass profile, and subsurface biomass maxima, resulting in the highest degree of overestimation of the true $\Delta F/F_m'$, and hence resulting in an overestimate of the maximum electron transport rate, $rETR_{max}$, often by as much as of 60% (Forster and Kromkamp 2004). The effect of this on estimates of depth integrated primary production will be discussed later when comparing fluorescence with other methods used to estimate primary production.

Vertical migration does not only influence the apparent value of $\Delta F/F_m'$, but it also influences the measured minimal fluorescence yield, F_o (Forster and Kromkamp 2004, Perkins et al. 2001, Jesus et al. 2006), which is used as a proxy of biomass (Barranguet and Kromkamp 2000, Honeywill et al 2002). It is thus not surprising that Jesus et al. (2006) observed that tidally induced vertical migration resulted in either an over estimation or under estimation of biomass dependent upon the time the measurements were made within the emersion period.

Recent work using migration inhibitors (Cartaxana et al. 2008, Perkins et al. in prep.) and engineered non-migratory biofilms (Jesus et al. 2006a,b; Mouget et al. 2008) have investigated these issues further and confirm that vertical migration and the concomitant "deep layer fluorescence" lead to erroneous estimates of quenching coefficients and overestimation of $\Delta F/F_m'$.

2.2 Stromatolites - the effect of "layered" biofilms

Stromatolites are perhaps an extreme example of a layered biofilm where sub-surface cells "interfere" with the fluorescence signal from surface cells and vice versa. The measurements become an integrated measurement of the sub surface cyanobacteria (Figure 2.3) and the surface cells, where diatom epiphytes can be present (Fig. 2.4, Perkins et al. 2007). Stromatolites consist of a microbial consortium

which trap ooids by extracellular polymers, mainly of cyanobacterial origin (Visscher et al. 1998, Reid et al. 2000). Reid et al. (2000) described 3 different developmental stages where the pioneering type 1 stromatolite has the lowest cyanobacterial diversity with *Schizotrix gebeleinii* as the dominant form. Type 2 is characterised by a micritic crust at the surface (often lacking diatom epiphytes, Kromkamp, pers. obs.) and in climax state type 3 the endolythic *Solentia* spp. bores into the ooids and fuses them together, giving the stromatolite its structure. Types 2 and 3 have the highest cyanobacterial diversity (Baumgartner et al. (2007). Figure 2.3 shows a typical example of a type 1 stromatolite (Kromkamp et al. 2007) with a clear subsurface layer, but the layer above also contain many cyanobacteria. This picture clearly highlights the difficulty when applying surface measurements using PAM fluorometry: the depth and thickness of the subsurface layer varies as well as the distance to the stromatolite surface. This means that quantitative analyses of both cyanobacterial biomass as well as effective quantum efficiency is very difficult as the apparent $\Delta F/F_m'$ and F_o is integrated over an unknown depth interval with varying biomass distribution. This problem can be partly circumvented by measuring on a cross section by putting the PAM fiberoptics parallel to the mat surface (Perkins et al. 2007). However, this will disrupt chemical and light gradients, especially of oxygen and light, have greatly influence photosynthetic activity (Kromkamp et al. 2007).

The following example demonstrates the problems with working with cyanobacteria (Fig. 2.5). RLCs were made on two sections of the stromatolite where the depth of the subsurface layer was respectively at 4 and 1 mm below the surface. The layer above the surface contained cyanobacteria (cf. *Schizotrix* sp.) but the biomass was substantially lower than in the subsurface layer. After the RLCs were performed, the surface layer was removed until the subsurface layer was exposed and

new RLCs were made. In both cases the measured rETR was much higher when the surface layers were not removed. Several explanations are possible: the cyanobacteria in the surface layer have a higher photosynthetic activity then cyanobacteria in the subsurface biomass maximum. Deeper in the stromatolite the oxygen concentration might be low, causing rapid inactivation of the stromatolite (Kromkamp et al. 2007, Perkins et al. 2007). This might be the case for the layer 4 mm below the surface but is unlikely for the layer 1mm below the surface. Alternatively, fluorescence from the deeper layers, where the irradiance is lower and where $\Delta F/F_m'$ will thus be higher than at the stromatolite surface, will cause an overestimation of true $\Delta F/F_m'$ of the cells at the surface. The degree of overestimation depends on the depth (and thus the irradiance) of the subsurface layer (which is unknown without destructive sampling) and on the biomass in the subsurface maximum relative to the cyanobacterial biomass in the upper layers. Because the RLCs on the exposed subsurface layers are rather similar, the contribution of "deep layer fluorescence" seems the most likely explanation for the higher rates of rETR measured at the stromatolite surface. A comparison with measurements on cross sections (Mouget et al. in prep) seems to corroborate this conclusion.

2.3 Deconvolution of depth integrated signals

The inherent photophysiological status of the microalgae comprising a biofilm can be estimated from depth integrated fluorescence measurements made on undisturbed samples. A method has been proposed to estimate the inherent light response through the deconvolution of light-response curves based on depth-integrated measurements (Serôdio 2004). This approach is based on the relationship existing between the depth-integrated fluorescence levels measured at consecutive

light levels of a light curve and the depth attenuation of the fluorescence signal, which results in a set of recursive equations to be applied to depth-integrated light curves. This approach has assumptions and limitations (Serôdio 2004; Jesus et al. 2006): i) it assumes an homogeneous photophysiological light response for all the microalgae; ii) assumes an exponential vertical attenuation of incident actinic light and emitted fluorescence, which implies a vertically homogeneous photic zone; iii) it requires the attenuation coefficients for downwelling actinic light and upwelling fluorescence to be known; iv) it does not account for changes in surface biomass during the construction of the light curve on intact biofilms. Nevertheless, numerical simulations using published values for actinic light and fluorescence have shown a significant reduction in the differences between depth-independent light curves and those deconvoluted from depth-integrated curves (Serôdio 2004).

3. Down regulation through Non Photochemical Quenching

3.1 NPQ and the Xanthophyll cycle in diatoms

Diatoms of MPB biofilms are submitted to an extreme light environment which includes exposure to both high visible and UV irradiances, and fast and unpredictable light fluctuations. Additionally, other environmental pressures (nutrient limitation, extremes in temperature and salinity, etc.) can slow down the photosynthetic machinery and create a situation where the photosynthetically converted light energy cannot be entirely used for metabolic purposes (see Chapters 11 and 12). In order to maintain their photosynthetic productivity at an optimal level by preventing photoinhibition (i.e. decrease in quantum efficiencies F_v/F_m and $\Delta F/F_m'$), diatoms need to acclimate to environmental changes through fast regulation of their photosynthetic activity (Lavaud 2007). Together with the PS II electron cycle

(Lavaud et al. 2002b), the non-photochemical quenching of chlorophyll fluorescence (NPQ) is believed to be one of the most important of these 'photoprotective' (or 'photoacclimative') mechanisms in diatoms (Lavaud 2007). Most of the diatoms of the MPB are able of vertical migration into the sediment as a behavioral photoprotective strategy in order to avoid excess light exposure at the surface (see above). However, vertical migration and NPQ appear to be complementary, NPQ induction may occur before the onset of the migratory response (Serôdio et al. in press, Underwood et al. 2005), although recent work using chemical inhibitors of migration and NPQ suggests that migration may be preferred to NPQ (Perkins et al. in prep.).

The NPQ mechanism is described in the Chapter 12 (paragraph 12.1). qE, the energy-dependent quenching, which is regulated by the build-up of a transthylakoid proton gradient (ΔpH) and the operation of the xanthophyll cycle (XC) (Lavaud and Kroth 2006), remains the best known component and plays a major role in the regulation of the diatom NPQ. The machinery triggering and controlling the NPQ amplitude and kinetics is now well known (Goss et al. 2006, Lavaud 2007). The major characteristic of NPQ in diatoms is its amplitude (Lavaud et al. 2002, Ruban et al. 2004) such that it can account for up to 90% of energy dissipation (Lavaud et al. 2002). Estuarine species of the MPB diatoms show a higher (up to 5 times higher than plankton species, Fig. 3.1) and faster (10 s induction) switch on/off of NPQ (Serôdio et al. in press; Serôdio et al. 2005, 2006, Herlory et al. 2007, Lavaud et al. 2007, Cruz and Serôdio 2008).

The mechanism of NPQ in diatoms shows other specificities regarding the pigments and proteins that are involved as well as their spatial organization (see Lavaud 2007). In particular, the xanthophylls cycle, which consists of the enzymatic de-epoxidation/epoxidation of the couple DD-DT, is employed (Fig. 3.2, Lavaud

2007). Accumulation of photoprotective diatoxanthin, DT, depends on the concomitant activity of two enzymes, a de-epoxidase and an epoxidase, the activity of which depends on the light intensity via the build-up of the transthylakoid ΔpH and the availability of co-factors (Fig. 3.2A).

The regulation of the XC in diatoms shows some striking peculiarities, the main one being the triggering of the diadinoxanthin, DD, de-epoxidase by a weak ΔpH (and thus a rather high lumen pH, Fig. 3.2B) (Jakob and Wilhelm 2001) so that the DD de-epoxidation already occurs at lower irradiances and shorter illumination times than in higher plants (Fig. 3.2B). Additionally, MPB diatoms can react to high light stress by accumulating large amounts of DD-DT (Rech et al. 2005, van de Poll et al. 2006, Schumann et al. 2007) in order to increase their photoprotection capacity via NPQ (Perkins et al. 2006, Schumann et al. 2007, Cruz and Serôdio 2008). All together, these differences in the regulatory components and mechanistic components of the NPQ process in diatoms have been suggested to ensure more flexibility and thus quicker response to the light environment (Lavaud 2007).

Field experiments have shown that the photoprotection ensured by both the XC and NPQ are essential for the MPB diatoms to maintain an optimal photosynthetic activity (Serôdio et al. 2005), even if not 100 % efficient (Serôdio et al. in press). Additionally, the differential ability of species to cope (including NPQ) with prolonged high light and UV (Waring et al. 2006) is believed to potentially control spatial distribution (Fig. 3.1) (Lavaud et al. 2007) as well as species succession within MPB biofilms (Tuji 2000, Serôdio et al. 2005, Underwood et al. 2005).

As described later in this chapter, RLCs have become a powerful approach to estimate the photosynthetic productivity and the photophysiology of MPB assemblages isolated from the field or directly in situ. Nevertheless, the assessment of

NPQ on MPB via RLCs is rendered problematic by the confounding effects on F_m and F_m' levels of 1) downward vertical migration of the cells in the sediment, 2) the light and fluorescence attenuation in the upper layers of the sediment, 3) the contribution of fluorescence originating in "deeper" layers with a concomitant lower irradiance, and 4) a sustained NPQ under prolonged darkness (see Serôdio et al. 2005, Jesus et al. 2006b). In order to solve this problem, Serôdio and co-workers proposed to assess NPQ following $\Delta\alpha_{RLC}$, which is the variation of the initial slope of RLC under high light exposure. α_{RLC} indeed linearly correlates with NPQ irrespective of measurement conditions, e.g. ex situ/in situ, summer/winter, low light/high light acclimation, etc. (Serôdio et al. 2005, 2006, in press, Cruz and Serôdio 2008) making it a good alternative to the classic calculation of NPQ as $(F_m-F_m')/F_m'$ (or $(F_0-F_0')/F_0'$, see Fig. 3.3A) (discussed in Serôdio et al. 2006). Additionally, it has recently been reported that RLC construction itself can generate rapid endogenous changes of the photosynthetic activity which in turn modulates chl a fluorescence emission, and can potentially affect the ETR measurement and subsequent derivation of photophysiological parameters (Perkins et al. 2006, Cruz and Serôdio 2008). Together with the redox state of Q_A, NPQ has been shown to be one the main endogenous mechanisms potentially disturbing chl a fluorescence emission (Perkins et al., 2006; Jesus et al., 2006b; Herlory et al., 2007).

There are a number of physiological/technical features which can strongly influence NPQ amplitude and kinetics, and subsequently the shape of RLCs in MPB communities dominated by diatoms: 1) the species composition (Perkins et al. 2002, Serôdio et al. 2005, Underwood et al. 2005), 2) the light/dark past history of the cells and hence the cells photoacclimation state (Perkins et al. 2006, Cruz and Serôdio 2008), 3) the accumulated light dose during the RLCs (Perkins et al. 2006, Herlory et

al. 2007 Cruz and Serôdio 2008), 4) the fluorometer used (Perkins et al. 2006, Cruz and Serôdio 2008). Figure 3.3 shows that, in the MPB diatom species *Navicula phyllepta*, the length of each irradiance step (10, 30, 60 or 140 s) can influence partly the level of NPQ induction (Fig. 3.3A), the profile of RLCs (Fig. 3.3B) and the subsequent determination of ETR_{max} as a function of the light history of the cells (Fig. 3.3C). However, it should be mentioned that this phenomenon is not always observed on intact MPB communities in situ as shown in Fig. 3.4.

3.2 NPQ in the dark

In diatoms, NPQ occurs not only during light exposure but also during prolonged darkness (several hours; Jakob et al. 1999, Consalvey et al. 2004, Serôdio et al. 2005). Dark NPQ is due to chlororespiration, the amplitude of which is especially high in diatoms (Dijkman and Kroon 2002, Lavaud et al. 2002b). This pathway allows electrons to flow from the NADPH, H^+ to O_2, both synthesized in light, via the plastoquinone (PQ) pool: it is the respiratory chain of the plastids (Kuntz, 2004). In diatoms, the chlororespiratory pathway is switched on very rapidly after the onset of darkness (Dijkman and Kroon 2002). Its amplitude and duration directly relates with the irradiance and duration of the former illumination through accumulation of reducing equivalents (Lavaud et al., 2002b), in other words it depends on the past light history of the cells (Cruz and Serôdio 2008). By transferring electrons through PQ, the chlororespiratory pathway generates in darkness the build-up of a transthylakoid ΔpH (Ting and Owens 1993). This dark ΔpH has been shown to be sufficient to activate the DD de-epoxidase and to drive the synthesis of DT, and the development of a large NPQ (up to 2) (Jakob et al. 1999, Jakob and Wilhelm 2001; Serôdio et al., 2005, 2006). This is only possible because in diatoms, as specified above (Fig. 3.2B), the DD de-epoxidase needs only a weak acidification of the lumen

to be activated, even though the rate of chlororespiration and subsequent change in the ΔpH are low (Jakob and Wilhelm 2001). Such a process would allow the cells to prevent photoinhibition during a subsequent sudden exposure to high light by keeping activated the dissipative function of the LHC system; an obviously adaptive advantage for the diatom species growing in a fluctuating light environment. Dark NPQ is even more physiologically relevant for diatoms of the MPB which can spend more than 18 h per day in the dark in the sediment before migrating to the surface and experiencing high light exposure (Serôdio et al. 2005).

As well as the NPQ which develops in light, the dark NPQ generates a methodological problem due to our inability to instantaneously measure the 'true' F_m (and F_0) level during dark-adaption of the cells in situ where their past light/dark history is usually unknown. Indeed, to achieve correct measurement of fluorescence parameters, complete Q_A oxidation and NPQ relaxation are required, which is usually reached after a short (15 min) dark-adaptation in controlled laboratory conditions but which might not be enough in situ (Perkins et al. 2001, Consalvey et al. 2004, Jesus et al. 2006a). Dark NPQ can easily quench F_m by at least 10-15 % depending on the diatom species (Jakob and Wilhelm 2001). When the cells are further exposed to a low irradiance (below 150 µmol photons m^{-2} s^{-1}, Mouget and Tremblin 2002) during the measurement following dark-adaptation (typically at the beginning of RLCs acquisition), the whole photosynthetic machinery is fully activated which 1) reoxidizes Q_A and the PS II, 2) dissipate the ΔpH and subsequently change the equilibrium of the XC, hence relaxing the fluorescence quenching (Consalvey et al. 2004, Serôdio et al. 2005, 2006). Hence, it is rather common to observe F_m' level transiently higher than the dark F_m level (Mouget and Tremblin 2002, Consalvey et al. 2004, Serôdio et al. 2005, 2006). As a consequence it significantly affects the

measurement and calculation of many fluorescence parameters. It can also significantly perturb the use of F_0 and its changes as a proxy for the dynamics of MPB diatom biomass at the surface and within the sediment (Consalvey et al. 2004, Jesus et al. 2006a). Solutions have been proposed to rule this problem out: 1) measurement of the 'true' F_m level in the presence of DCMU which is only applicable in controlled laboratory conditions (see Chapter 12, paragraph 12.4), 2) short exposure to low dose of far-red or low light instead of dark-adaptation in order to reoxidize PS II and dissipate the ΔpH and NPQ (Consalvey et al. 2004, Jesus et al. 2006a), 3) use $F_m'_{,m}$, the maximum F_m' value measured as the 'true' F_m, instead of the dark F_m level (Serôdio et al. 2006, in press, Cruz and Serôdio 2008).

4. The quantification of the microalgal biomass using fluorescence

The quantification of the microalgal biomass of microphytobenthos biofilms is a methodological challenge, due to the thinness of the sediment photic zone, the large horizontal heterogeneity, and the rapid changes in microalgae near the surface due to vertical migratory movements. Serôdio et al. (1997) was the first study to investigate the possibility of using in vivo chlorophyll fluorescence to non-destructively quantify the microalgal biomass of microphytobenthos biofilms. They experimentally established a linear relationship between sediment chlorophyll a (chl a) content and minimum fluorescence yield F_o, showing that large F_o variations in the dark represented changes in the amount of microalgae at the sediment surface, as the result of cell vertical migratory movements. The use of F_o as a biomass proxy was shown to be preferable to other fluorescence parameters such as F_m or F_m' because it varied the least with previous light history, temperature and microalgal group. F_o was shown to allow the estimation of the miroalgal biomass present in the photic zone of the

sediment (defined as ‘photosynthetically active biomass’; Guarini et al. 2000, Honeywill et al. 2002) but also of the microalgal biomass in the photic zone weighted by its contribution to depth-integrated photosynthesis (defines as ‘producitve biomass’; Serôdio et al., 2001). This method allowed to overcome the operational difficulties with previously used methods, based on destructive and time-consuming procedures, and introduced considerable operational advantages, including the possibility to obtain repeated measurements in the same sample over time, without any physical disturbance of the sediment-air or –water interface, and allowing the concurrent measurement of other variables in the same sample (Serôdio et al. 1997).

However, the determination of F_o in MPB biofilms is not without problems and has been the source of significant discussion (e.g. Consalvey et al. 2004, Jesus et al 2006b,c). These problems are related to MPB vertical movements during the dark adaptation (DA) period and to problems concerning the presence of NPQ in the dark (exhibited by diatom dominated biofilms). The determination of fluorescence parameters requiring dark-adaptation (F_o, as well other parameters and indices such as F_m or F_v/F_m) requires that PSII reaction centres and Q_A be in their fully oxidised form (Schreiber et al. 1986). To achieve this state it is conventional to place samples in full darkness until a dark adaptation steady state is reached. However, dark adapting microphytobenthic biofilms present a number of specific problems unique to this type of community.

Microphytobenthic biofilms are know to exhibit a behavioural photo-regulation mechanism where microalgae migrate vertically within the sediment matrix as a response to changes in ambient light in order to maintain an optimum light environment and avoid photo-inhibition (e.g. Perkins et al. 2002; Jesus et al. 2006b,c). This response to changes in ambient light can hinder the determination of F_o if

vertical migration occurs during the dark adaptation period necessary for Q_A and PSII re-oxidation. This has been recognized as a potential problem since the first studies using fluorescence (e.g. Serôdio et al. 1997) and short adaptation periods have been suggested as alternatives. Several forms of determining the minimum fluorescence yield have been tested, depending on the length of dark-adaptation used (F', F_o', F_o^2, F_o^5, F_o^{10}, F_o^{15}; the superscript number denoting the duration of the dark period in minutes). By far, the most commonly used parameter is F_o^{15} (Kromkamp et al,1998; Underwood et al. 1999; Barranguet and Kromkamp 2000; Perkins et al. 2001; Hagerthey et al. 2002; Honeywill et al. 2002; Underwood 2002; Consalvey et al. 2004; Defew et al. 2004; Jesus et al. 2006b,c), followed by 5 min (Serôdio et al 1997, 2000, 2001, 2003; Jesus et al. 2006b,c) and 2 min (Serôdio et al. 2006, 2007 and 2008). However, the 15 min of DA can be excessive in some biofilms inducing significant downward migration during that period (e.g. Jesus et al. 2006a, b and c), suggesting that shorter time periods might be preferable. In fact, Jesus et al. (2006c) compared the relationship between chl a with F_o', F_o^5 and F_o^{15} and found no significant differences from F_o' to F_o^{15} in muddy sediment assemblages, suggesting that light history had little or no effect in the epipelic assemblages and that no DA was necessary for a good relationship between chl a and fuorescence. However, in sandy sediments there was evidence of a light history effect and a 5 min DA period was necessary to remove this effect. Other authors (e.g. Serôdio et al. 2007) have found that shorter DA times (2 min) might be even better than 5 min for the determination of photosynthesis fluorescence indices that require the input of a minimum fluorescence yield parameter.

Intertidal biofilms exhibit the additional problem of not showing an homogeneous behavioural response to light stimulus throughout the tidal cycle, i.e.

biofilms close to the beginning of the emersion period will tend to increase F_o values during the DA period as a result of cells migrating to the sediment surface, and measurements close to the end of the emersion period will be very sensitive to darkness and cells will migrate downwards quickly over the DA period. Although, this does not seem to be the case in all estuaries (e.g. Kromkamp et al,1998; Barranguet and Kromkamp 2000; Hagerthey et al. 2002; Honeywill et al. 2002;). To reduce this problem it was proposed that measurements are taken closer to the middle of the emersion period and that a low light or far-red treatment is used instead to the dark treatment (Jesus et al. 2006b). It is not clear why low light and far-red light work similarly but both treatments have to be applied at a reduced photon flux quantity to work properly. Thus, it is possible that this reduced photon flux promotes the dissipation of the chlororespiration trans-thylakoid proton gradient (pH) exhibited by diatoms in the dark (Ting and Owens, 1993, Dijkman & Kroon, 2002; Lavaud et al., 2002). The reduction in the ΔpH promotes the exoxidation of diatoxanthin into diadinoxanthin. Therefore, decreasing the NPQ caused by diatoxanthin presence in the dark (Jakob et al. 1999, Jakob and Wilhem 2001). Another advantage of low light is that it seems to promote the presence of cells at the surface thus reducing the problem of migration downwards that occurs during the dark adaptation period (Jesus et al. 2006b).

5 Calculation of electron transport rate: ETR v rETR

5.1 Multiple and single turnover methods

The rate of electron transport by PSII depends on the amount of light absorbed by the antenna of PSII and the efficiency at which the absorbed light by PSII is used by the reaction centers (RCII) to drive charge separation. Basically two methods are used which are both based on the light doubling method originally proposed by

Bradbury and Baker (1981) which we will call the Multiple- turnover (MT) method and the single turnover (ST) method and which relate to the description of the pulse-amplitude modulation principle by Schreiber et al. (1986) and the pump and probe method (Falkowski et al. 1986b) respectively. The differences between these two approaches have recently been reviewed by Kromkamp and Forster (2003).

5.2 The MT-method.

The MT-method is usually used by scientists using the PAM family of fluorometers which uses a multiple turnover flash to measure the quantum efficiency of PSII. Because the flash duration used to measure the maximum fluorescence (F_m) is relatively long (50ms - 1 sec) it allows for multiple charge separations during the flash and will thus lead to fully reduced Q_A, Q_B and PQ-pool. When the PSII effective quantum efficiency ($\Delta F/F_m'$) is measured the absolute rate of electron transport per unit area (ETR^A) can be calculated as follows:

$$ETR^A = E \bullet A_{II} \bullet \Phi_{RC} \bullet \Delta F/F_m' \qquad (1)$$

A_{II} is the fraction of the incident light (E) which is absorbed by PSII. Φ_{RC} is yield (in electrons) of reduced Q_A per trapped photon, i.e. the maximum quantum yield of photochemistry within PSII, and it is usually assumed to be equal to 1 (Kolber and Falkowski 1993). Note that ETR^A is expressed in µmol electrons s^{-1} m^{-2} of surface area. When working with higher plants it is assumed that leaves absorb approx. 85 % of incident light, and about half of this is partitioned to PSII. Assuming that Φ_{RC} is close to 1, eq. (1) can be rewritten as:

$$ETR^A \approx 0.43 \bullet E \bullet \Delta F/F_m' \quad (2)$$

Often the fraction of absorbed light is determined by measuring the transmittance of light through a piece of macroalgal thallus using a light sensor (Beer et al. 1998, Longstaff et al. 2002). Implicit in this assumption is that the measured signal is equivalent to the integrated fluorescence yield over the entire path length, an assumption which might not be met, certainly when several cell layers are involved. The artefacts associated with this assumption were discussed in chapter 3 where the effects of "deep layer" fluorescence are described.

When working with optically thin suspensions of phytoplankton or unicellular algal cultures a slightly different approach is taken: in this case the ETR is usually calculated per mg chl a (µmol e^- (mg chl a)$^{-1}$s^{-1}):

$$ETR = E \bullet a'^*_{PSII} \bullet \Delta F/F_m' \bullet \Phi_{RC} \quad (3a),$$

Where a'^*_{PSII} is the optical absorption cross section of PSII (here expressed in m^2 (mg chl a)$^{-1}$, which is the product of the cross section of a single PSII unit and the number of PSII per mg chl ($a'^*_{PSII} = a^*_{PSII} \bullet n_{PSII}$)). As it is rather difficult to measure the absorption cross section of PSII it is normally assumed that a'^*_{PSII} is half the optical cross section of the cells (a^* m^2 (mg chl a)$^{-1}$, i.e. it is assumed that 50% of the absorbed light is funneled to PSII and the other half to PSI). Below we will discuss this assumption. Thus, assuming Φ_{RC} equals 1 we can rewrite eq. 3a as:

$$ETR = E \bullet a^* \bullet 0.5 \bullet \Delta F/F_m' \quad (3b).$$

The optical cross section can be easily determined using a spectrophotometer equipped with an integrating sphere, i.e. by using the filterpad method for natural phytoplankton which requires concentration on a filter before the absorption measurements can be made (Tassan and Ferrari 1998, Simis et al. 2005).

5.3 The ST-method

Researchers using a saturating single turnover flash to measure F_m' usually take the approach developed by Falkowski et al. (1986b) and Kolber and Falkowski (1993), based on the development of the pump and probe fluorometer, which was followed up by the Fast Repetition Rate Fluorometer (FRRF) (Kolber et al. 1998). Here the rate of photosynthetic electron transport is described as follows:

$$ETR = E \bullet a^*_{PSII} \bullet n_{PSII} \bullet q_P \bullet \Phi_{tm} \bullet \Phi_{RC} \bullet f \quad (4)$$

Here a^*_{PSII} is the optical cross section of a single PSII unit (m^2/mol PSII) and n_{PSII} is the number of PSII units per mg chl a, usually to be assumed equal to 0.002 (thus assuming 500 chl a molecules per PSII (Falkowski 1981, Kolber and Falkowski 1993). The photochemical quenching coefficient q_P (moles electrons transferred per mole photons absorbed by PSII) reflects the proportion of oxidized PSII centers and is often used as a proxy of the number of open reaction centers. This is, however, only true when the PSII centers are not connected (Kramer et al. 2004). The trapping efficiency Φ_{tm} is the efficiency at which trapped photons in the pigment bed are transferred to an open RCII and f is the fraction of functional PSII centers. The product of the optical PSII cross section and the trapping efficiency equals the effective PSII cross section (σ_{PSII}, units m^{-2} (mol PSII)$^{-1}$):

$$\sigma_{PSII} = a^*_{PSII} \bullet \Phi_{tm} \quad (5).$$

As the FRRF can measure the functional cross section σ_{PSII} from the rise of F to F_m during the induction flashlet sequence, eq. 4 can be rewritten as

$$ETR = E \bullet \sigma_{PSII} \bullet n_{PSII} \bullet q_P \bullet \Phi_{tm} \bullet f \quad (6).$$

Eq. 6 is the one that is proposed by Kolber and Falkowski (1993) and Kolber et al. (1998) for use with the pump and probe and FRR fluorometer. The fraction of inactive centers (f) was measured as $\Phi_{sat}/0.65$, where Φ_{sat} is the maximum PSII efficiency measured and 0.65 the assumed maximum PSII efficiency of healthy cells without non-functional PSII centers. Kromkamp and Forster (2003) argued that this factor should be omitted because non-functional PSII centers will affect the level of F_o', and this is already incorporated in the value of q_P.

5.4 Assumptions and uncertainties.

Fraction of light absorbed by PSII. Both the MT as well as the ST method usually use a number of a-priori assumptions. When using the MT method to calculate ETR it is necessary to know which fraction of the absorbed light is funneled to PSII. Using a combination of optical and biophysical techniques. Suggett et al. (2004) tested the hypothesis that about 50% of the light is absorbed by PSII. For a large range of species (diatoms, green alga, haptophytes and a cryptophyte) the fraction of light absorbed by PSII varied between 0.48 and 0.58, justifying this assumption. However, in the pelagophyte *Aureococcus anophagefferens* the PSII-antenna

absorbed about 36% of the light and in two cyanobacterial *Synechoccocus* species PSII absorbed 25-32% of the total light. Interestingly, the fraction of light absorbed by PSII was independent of the growth irradiance. A similar approach was taken by Johnsen and Sakshaug (2007), but they used a slightly different scaling procedure to match the fluorescence excitation spectra to the absorption spectra in order to arrive at the fraction of light absorbed by PSII: their results show that 48-88% of the light absorbed by the photosynthetic pigments was absorbed by PSII, which is generally higher than the estimates obtained by Suggett et al. (2004). Whether this difference is entirely due to methodological question or to growth conditions and different algal species remains an open question, but clearly this topic requires further research.

Estimates of the number of PSII. When using the ST protocol the absorption is quantified by multiplying the measured functional cross section σ_{PSII} with the n_{PSII}. This latter factor is also rather difficult to measure, certainly using field material, and for this reason it is assumed that n_{PSII} equals 0.002, i.e. a PSII contains 500 mol chl a $(\text{mol PSII})^{-1}$ (Kolber and Falkowski, 1993), a value based on the determinations by the photosynthetic unit (PSU) size by Mauzerall and Greenbaum (1989). Assuming that a PSU contains 4 RCII it can be calculated from the data presented by the references in Table 1 that generally n_{PSII} varies between 500-725 mol chl a per mol PSII, although lower values have been reported for *Isochrysis galbana*.

Photoacclimation generally results in more n_{PSII} when the cells are grown in low irradiances (Table 1). Interestingly, Kromkamp and Limbeek (1993) observed that when the marine diatom *Skeletonema costatum* was grown in fluctuating light simulating vertical mixing, this resulted in smaller but more n_{PSII}. This makes sense as it allows the cells to both harvest the same amount as light as with large PSU, but it will result in a higher rate of maximal photosynthesis. Recently Suggett et al. (2004)

compared estimates of n_{PSII} obtained using the ST turnover oxygen measurements with a those obtained from a biooptical: $n_{PSII} = \sigma_{PSII}/a^*_{PSII}$: they observed a linear 1:1 relationship between both methods and for most eukaryotic algae n_{PSII} varied between 500-600 mol chl a per mol PSII. The pelagophyte *A. anophagefferens* had a larger n_{PSII} and cyanobacteria seem to contain smaller n_{PSII} (240-280 mol chl a per mol PSII).

Uncertainties in σ_{PSII}. Although the FRRF technique allows estimation of the measurement of the functional PSII cross section, it is necessary to stress that LED's (mainly blue) are used to induce the fluorescence induction curve from which σ_{PSII} is estimated. Without spectral correction of the effective absorption of the FRRF in relation to the underwater light field, this may lead to an overestimation of σ_{PSII} (Suggett et al., 2001).

5.5 Calculation of ETR in microphytobenthos studies

Most research measuring ETR on MPB or macroalgae have used a PAM-type fluorometer because a commercially available FRR-type fluorometer (such as Chelsea's Fast^{Tracka} or Satlantic's FIRe) that are able to measure σ_{PSII} , have not been available (RandD versions of both instruments have however been used for coral reef research). As is clear from the above section on the MT-protocol, calculation of absolute rates of PSII electron transport requires knowledge of incident irradiance and the optical absorption cross section, and this is exceedingly difficult when working on benthic biofilms. For this reason often the relative rate of ETR (rETR) is calculated as $\Delta F/F_m' \bullet E$. Because the value of a* will change as a result of photoacclimation (time scales of change are hours-days because they related to de-novo synthesis or break-down of pigments) it is not always possible to compare rates of rETR between publications.

Morris and Kromkamp (2003) cultured the benthic diatom *Cylindrotheca closterium* at two different growth rates and compared the relationship between ETR and oxygen evolution at a range of different temperatures during steady state growth. In general they observed that the relationship between ETR and oxygen evolution was not very sensitive to short-term changes in temperatures. However the relationship of rETR versus oxygen evolution was rather different between low and high growth rate, but when they examined the relationship on the basis of absolute ETR, the differences were minor. This was due to the a* values of the two cultures being different between the two growth rates. Nevertheless, often changes in rETR reflect changes in absolute ETR (Fig. 5.1): three out of the four different algal species showed a similar pattern in change in $rETR_{max}$ and absolute ETR_{max} (expressed per cell), despite large changes occurring in the photosynthetic physiology after transfer from replete to a P-free medium. The exception was *Emiliania huxleyi* which showed an unexpected increase per cell, because the optical absorption cross section per cell increased. If the absolute ETR was expressed per unit chl a all cells showed a good correlation between the changes in rETR and ETR (data not shown). This suggests that with some care it maybe possible to deduce changes in photophysiology from relative rates of ETR, but definitely more research is needed to confirm this.

A possible way to obtain MPB cells in order to measure a* spectrophotometrically would be to use the lens tissue method: however, this will only select for (a fraction) of the migrating species and the observed a* value might not be representative for the total MPB community. In order to avoid this problem Morris et al. (2008) reconstructed a* from HPLC pigment analyses using the procedure of Bidigare, (1990) and compared the absorption values to the ones measured using the filterpad method. From this it was concluded that the package

effect reduced the maximum absorption spectra obtained from the HPLC data by about 30%, in line with other studies on MPB (Mercado et al., 2004) and phytoplankton (Berner et al., 1989; Johnsen and Sakshaug, 2007) although Nelson et al. (1993) reported that less than 25% of the phytoplankton in Californian coastal waters showed a measurable package effect. Using the reconstructed and measured a* values Morris et al. (2008) demonstrated that the PAM derived quantum efficiencies for C-fixation (Φ_C) on both intact diatom biofilms as on sediment slurries with defined optical conditions matched the measured Φ_C very well. Only when growth rate of the diatom biofilm slowed down when the biomass reached an apparent steady state did the PAM derived Φ_C of the biofilm overestimate the measured Φ_C of the sediment slurry, whereas Φ_C of the slurry matched the measured Φ_C. Most likely the overestimate of the true Φ_C was caused by fluorescence derived from deeper layers (see above). This suggest that quantification of ETR on MPB biofilms is possible using reconstructed a* values from HPLC derived pigments, provided the diatoms in the biofilm are actively growing and the biofilm does not reach a high biomass.

6. Light response curves

6.1 A brief overview of methodology

Light response curves (the photosynthesis – irradiance curve following Falkowski and Raven (1997)) are used to determine a range of photophysiological and productivity parameters. Incremental increases or decreases in the actinic light environment (PAR, usually applied by the flourometer in use) are applied, with measurement of the quantum efficiency ($\Delta F/F_m'$). The well known and accepted equations for calculation of ETR are applied and the resulting values plotted as a function of PAR. The result is a saturating curvilinear response, encompassing a near

linear light limited phase, a light saturated phase and then and, in some instances, a third phase which is not always present and often attributed to down regulation/photoinhibition. Various methods of curve fitting have been applied to this curve, from which physiological parameters can be derived, including the method of Eilers and Peeters (1988) followed by curve fitting such as that following the Nelder-Mead model (Press et al., 2003). Parameters derived are usually:-

1. $rETR_{max}$ – the maximum relative electron transport rate when light becomes saturating.
2. α - the maximum light use coefficient
3. E_k - the light saturation coefficient, calculated as $rETR_{max} / \alpha$
4. β - the coefficient of down regulation / photoinhibition

These parameters, except β, along with the PAR at which saturation occurs (Es) can be calculated using the parameters derived from various models such Eilers and Peeters (1988).

The application of the methodology to benthic biofilms has been reviewed in Consalvey et al. (2005), but here it will be discussed principally to compare the three main types of light response curves: steady state light curves (SS), rapid light curves (RLC) and non-sequential light curves (NSLC). Light curve parameters ($rETR_{max}$, α etc.) are mostly interpreted in similar ways for all light curves, except for the coefficient β. In SS light curves the decline in ETR indicated by the value of β is attributed to photoinhibition, whereas in RLCs β is a measurement of down regulation as the short duration of the light curve is not thought sufficient to induce photoinhibition (White and Critchley 1999). The other parameter worth mentioning here is $rETR_{max}$, where the subscript (r) preceding ETR denotes whether the measurement is of "relative" electron transport rate, i.e. in the absence of any

measurement of the light absorption coefficient a* (Sakshaug et al. 1997, Beer et al. 2001, Forster and Kromkamp 2004, Perkins et al. 2006). In most instances measurement of a functional value of a* is highly problematic for benthic biofilms, due to interference from the sediment matrix and the time required for measurement (see elsewhere in this chapter for more detail). As a result the majority of work has used rETR and hence determined $rETR_{max}$. Data are often considered to be comparative as a result of this relative measurement, as opposed to absolute values for comparison to other measurements of productivity (e.g. ^{14}C, O_2 described later in this chapter).

6.2 Steady sate light curves

Steady state (SS) light curves are those where the operational fluorescence yield (F or F') is allowed to reach a stable value after each incremental increase / decrease in PAR (Falkowski and Raven 1997). Thus the effects of Q_A reduction/oxidation and NPQ induction/reversal are allowed to reach completion for the light environment applied. Once this has been achieved the saturating pulse is applied to determine the rise in fluorescence yield to F_m' and hence calculation of $\Delta F/F_m$'. SS light curves are often considered to be a measurement of the potential photophysiology of the cells at the time of measurement, as opposed to their actual operational photophysiology at that time. Hence, they reflect the capacity for photosynthesis and for this reason allow comparison between SS light curves of different species and under different environmental conditions. SS light curves can thus be used for examination of phenotypic and genotypic adaptation. For example, the effects of light dose history prior to the light curve are greatly changed, or possibly negated, due to the photoacclimation during each step of the light curve. Obviously some aspects of prior photoacclimation will remain, such as changes in

pigment bed which take longer to be modified by the cells. In fact the time to reach steady state is a function of light history, e.g. the rate and magnitude of NPQ induction / reversal is a function of previous light dose history. This time period is therefore highly variable, and can be in the order of several minutes or longer. This has several drawbacks when applied to benthic biofilms:-

1. The long duration of the light curves (up to 40 minutes for an 8 step light curve[1]) often makes replication of measurements impossible.
2. Investigation of temporal or spatial variation is prohibited by the long time period spent obtaining a single set of measurements.
3. Changes in biofilm surface community may occur over this period of time due to microcycling (e.g. Kromkamp et al. 1998) and positive or negative phototaxis (e.g. Serôdio et al. 2006) induced by the applied PAR, as well as possible diel and tidal patterns in vertical migration (see elsewhere in this chapter), hence measurements at incremental light steps may be made on different cells.

As a result, SS light curves are usually only used with benthic biofilms to determine light parameters in theoretical investigations, rather than in determination of absolute values of light curve parameters. However even in these situations the issue mentioned in Point 4 above can make the light curve largely uninformative, or at least difficult to interpret due to the rapid changes in community structure between light steps. Perkins et al. (2002) demonstrated the complete change in surface community in a benthic biofilm using high resolution fluorescence imaging (HRFI) in an 8 minute light curve, changes being observed in surface diatom taxa and finally a complete

[1] Walz fluorometers such as the Diving-PAM and Water-PAM are programmed to have 8 step light curves. However other manufacturer's fluorometers differ and can allow the number of steps to be dictated by the user.

change to a *Euglena* spp. dominated surface biofilm (Fig. 6.1). As a result SS light curves are often limited to work using cultures of cells or engineered biofilms; in both case migration is inhibited, or preferably prevented (e.g. Jesus et al. 2005; Perkins et al. 2006; Mouget et al. 2008).

6.3 Rapid Light Curves

The definition of a RLC is somewhat difficult: when does a light curve become "rapid"? For example Kromkamp et al. (1998) used light curves that were not SS curves, but had light steps of 2 minute duration. This followed on from the first "rapid" light curve work by Schreiber et al. (1994) and Schreiber et al. (1997), the former using 5 minute steps, the latter using truly rapid light curves of 10 seconds. It is the opinion of the authors that to be a true RLC, light steps should be of less than 60 s duration. Using this definition, RLCs were not applied to benthic biofilms until the work of Serôdio et al. (2005), with work on seagrass reviewing the use of RLCs by Ralph and Gademann (2005) in the same year. The methodology was further investigated using benthic diatom cultures and microphytobenthos suspensions (Perkins et al. 2006, Serôdio et al. 2006, Herlory et al. 2007, Cruz and Serôdio 2008) and is now an accepted method for field and laboratory measurements of operational photophysiological state.

In some cases RLCs can be considered to be a compromise between SS light curves and practical limitations of data acquisition and replication. This however is an error as RLCs measure an entirely different photophysiological state, namely the operational photophysiology at that time. The duration of light steps used is often a compromise between practical time limitations and obtaining the required measurements. The longer the duration of each light step, obviously the longer the

cells exposed to this new PAR will have to photoacclimate and the measurement becomes an intermediate between a RLC and a SS light curve. Indeed many workers now prefer RLCs between 10 and 30 s at each light step, with 60 s considered too long due to effects of photoacclimation during the light curve. Also an important consideration, as discussed above, is that the longer each light step, the longer the cells will have to migrate in response to the applied PAR or as part of diel and tidal patterns of vertical migration, hence altering the community structure being investigated during the light response curve itself.

Typically RLCs are of 10, 20 or 30 s at each light step and often have 8 incremental steps[1] after an initial measurement in darkness (dark adaptation prior to the light curve is discussed below). Thus RLCs may be as short as 80 to 240 s. However migration and photoacclimation will still occur to some extent, with cells migrating vertically at speeds such as 0.17 – 0.28 μm s^{-1} (Consalvey et al. 2004). Serôdio et al. (1997) estimated the effective measurement depth using fluorescence to be 270 μm and Kromkamp et al. (1998) estimated detectable fluorescence measurements from only 150 μm sediment depth. Both these estimates are well within the depths to which benthic diatoms migrate, demonstrating the ability of the surface community under investigation to change over even a RLC. Furthermore, Perkins et al. (2006) reported detectable photoacclimation by cultures of benthic diatoms in RLCs with light steps of 10, 30 and 60 s duration. In this case the level of photoacclimation increased as a function of the increase in light step duration, especially for light curves with incremental increases in, as opposed to decreases in, PAR (Fig. 6.2). They further concluded that RLCs with decreasing PAR light steps were preferable to minimise this effect and that light steps of 20 to 30 s duration were preferable. However if samples have been dark adapted prior to the light curve, then back

pressure on the electron transport chain due to the time required to reactivate dark reactions, e.g. RUBISCO activation, could result in under estimation of $\Delta F/F_m'$. It should also be noted that decreasing light steps are not appropriate for SS light curves in which reversal of NPQ is required.

Perkins et al. (2006) indicated that 10 s was not thought to be an acceptable duration due to limitations of measurements by the flourimeter used (Walz Diving-PAM control unit). When cells had been exposed to high light, their rapid induction of NPQ to quench the fluorescence yield after the incremental increase in PAR was such that there was a considerable change in operational fluorescence yield (F) between measurement of F and application of the saturating pulse and subsequent measurement of F_m'. Where this happened, F_m' was under estimated relative to F causing under estimation of $\Delta F/F_m'$ and hence the calculated value of ETR.

While this question demands for further research, it is clear that the most appropriated light step to be used in each case will depend on the relative benefit of avoiding the mentioned artefact when applying 10 s, and of the confounding errors introduced by increasing the light step to 20s, namely short-term photoacclimation and migratory responses during the construction of the light curve. For example, the detection of diel rhythms in the photoacclimation status of microphytobenthos using RLCs has been shown to be possible when applying short light steps of 10 or 20 s, whilst becoming almost undetectable when using light steps of 40 s due to the rapid light-activation of dark-acclimated samples (Serôdio et al. 2005).

Despite the mentioned potential problems, incremental RLCs using 10 s light steps were shown to allow to characterise the steady-state photoacclimation status of samples acclimated to a wide range of ambient irradiances, through the estimation of the light-saturation parameter E_k from RLC parameters (Serôdio et al. 2006, Cruz and

Serôdio 2008). These studies have also demonstrated that such short RLCs could effectively 'capture' the steady-state photoacclimation status formed prior to the RLC. RLC parameters responded to ambient irradiance (Fig. 6.3) in a well-defined way, with the initial slope of RLCs decreasing with ambient irradiance (related to the build-up of NPQ; see section on XC), and maximum ETR increasing with ambient irradiance. As a consequence, RLCs could be related to steady-state light curve parameters, with significant correlations being found between the initial slopes of both light curves for samples acclimated to lower ambient irradiances, and between the maximum ETR of both curves for samples acclimated to higher ambient irradiances (Serôdio et al. 2006).

In their application to benthic biofilms, RLCs have distinct benefits over traditional SS light curves. They minimise changes in community structure due to migration, as well as reducing methodologically induced photoacclimation to modify the photoacclimation state of the cells under investigation. However the authors suggest that care needs to be taken in their interpretation as they are comparative measurements that should only be compared between studies using the same methodologies. Data are only comparable so long as the duration of the incremental light steps used are the same. It should also be noted that the magnitude of light level used for each light step will also result in variation between studies; photoacclimation will be proportional to the total light dose experienced during the light curve and measurements with higher light levels will induce more photoacclimation over the duration of the light curve.

It is worth mentioning at this point the issue of dark adaptation prior to the light curve itself. In many studies light curves are preceded by a short period of dark adaptation. This is usually to obtain an approximate measurement of the maximum

fluorescence yield F_m for calculation of parameters such as NPQ (where NPQ = (F_m-F_m')/F_m', see elsewhere in this chapter). However it can also be argued that such dark acclimation modifies the photoacclimation state of the cells investigated and, if of sufficient duration, dark acclimation can induce vertical migration (Jesus et al., 2006). However work by Kromkamp et al. (unpublished) indicated that a dark adaptation period of 1 minute prior to the light curve did not affect the RLC data obtained in a series of light curves logged over an emersion period. It is probable that the effect of the dark period is proportional to the photoacclimation state of the cells, and hence on the photodose (light history effect) experienced by the cells, as well as the propensity for the cells to migrate as a function of changes in light environment. It is suggested that, to standardise methodologies and hence increase comparative capability, dark adaptation should be avoided prior to light curves. Comparative values of F_m and hence calculation of parameters such as NPQ may still be achieved as in most cases fluorometer programming results in the first light curve steps being in darkness and at low light. The former replaces the dark adapted measurement, and Jesus et al. (2006) indicated that, for benthic diatoms which retain NPQ in the dark, low light reverses NPQ more efficiently than darkness, hence giving a value of F_m' which exceeds F_m in some instances (see elsewhere in this chapter). However, as with most methodologies, the use of dark adaptation will depend on what is being investigated, and in some studies reversal of NPQ prior to the light curve may be required.

6.4 Non-sequential Light Curves

The last form of light curve to be discussed here are NSLCs, in which each light step is applied to a separate culture sample or biofilm sample/area that has not been used for any previous measurements as part of the light curve (Perkins et al.

2006, Herlory et al. 2007). For example, the fluorometer probe is placed over one (sub)sample for the selected light step duration, at the end of which ΔF/Fm' is obtained. The probe is then moved to the next (sub)sample for the next light step of the light curve. There is no requirement here for the measurements to be sequential, but for this methodology dark adaptation prior to the light curve is not possible. In most cases this is a theoretical light curve as it requires an extensive set of replicate samples (e.g. cultures) or, in the case of field measurements, a substantially large area of biofilm which is considered to be homogeneous enough to allow a large number of replicate measurements. This method should therefore almost totally remove the photoacclimation induced by the light curve and, with enough replication, can be considered to be measuring the true operational photoacclimation state of the cells. The disadvantage is the number of replicates required.

Perkins et al. (2006) applied this method to cultures of benthic diatom cells, principally the species *Navicula phyllepta*. Despite using different culture sub samples for each light step, photoacclimation still occurred, with $rETR_{max}$ proportional to the increasing length of each light step. Notable however was that down regulation was observed at irradiances above the point where the curve saturated when light steps were of short duration (e.g. 10 and 30 s), and also that steady state light curves did not saturate at all and showed the highest $rETR_{max}$ values (Fig. 6.4). This demonstrates the rapid rate of induction of photoacclimation processes during the light curve steps themselves. Also reported by Perkins et al (2006) was the effect of light history, when cultures incubated at high light level (400 μmol m^{-2} s^{-1} PAR) prior to the light curve measurements did not saturate or show down regulation, but cultures incubated at low light (25 μmol m^{-2} s^{-1} PAR) showed both saturation and down regulation for 10 and 30 s light curves, saturation for 60 s curves, but no saturation for steady state curves.

The rate and level of photoacclimation processes was therefore proportional to light history and the length of the light curve steps used. An important point that should be mentioned at this point is that the light curves referred to in Perkins et al. (2006) used rETR. It would be expected that as photoacclimation increased, a higher level of NPQ induction would result in a lower effective ETR. Thus if the cross section of PSII had been measured to determine the "true" ETR as opposed to rETR, the ETR_{max} may decrease as a function of increasing light step duration. This clearly needs further investigation, although the measurement of PSII cross section is difficult for benthic biofilms during light curves.

6.5 Light Curves Summary

The use of steady state light curves has been a powerful tool in the use of fluorescence to investigate photophysiology of photoautotrophs, but due to the long duration required for measurement to be achieved is not practical for the use with benthic biofilms, especially those exhibiting rapid migratory activity in response to changes in light environment or as part of diel and tidal rhythms. It is suggested here that RLCs of 10-30 s duration with no prior dark adaptation step should be utilised, the particular light step to be applied being dependent on the previous evaluation of the relative magnitude of the errors introduced by (i) photoacclimation and (ii) migration during the RLC, and (iii) fluorometer software, factors varying with sample composition and light history, light levels applied during the RLC, and instrument characteristics. Furthermore, RLCs starting at high light and proceed to lower irradiance may represent a advantageous alternative to minimize the effect of cumulative light dose on the PSII quantum efficiency, although systematic comparative studies are still missing. Having said this, this would require a change in

the setup of the automated LC used to be made with Walz-PAM fluorometers, as currently the models mostly used in the field (minPAM, divingPAM) can only make LC from low to high light. Obviously this does not rule out further work in which different durations of light step are used to investigate photoacclimation etc., indeed this is highly recommended by the authors, especially with regard to the use of RLCs *in situ*.

7. Comparison of fluorescence with other methodologies

In section 5 we described how absolute rates of electron transport can be calculated. However, ETR is not the common currency in primary production studies. Photosynthetic rate measurements in aquatic organisms usually imply recording of either O_2 emission (e.g., oxymetry, mass spectrometry) or CO_2/HCO_3^- uptake (e.g., ^{14}C labelling, infra-red gas analysis) and in the marine environment as well as in ecosystem models C is the most often used. A comparison between ETR and the rate of oxygen evolution or C-fixation (P^B) can be obtained from a comparison of both methods: when P^B is plotted as a function of ETR the regression coefficient (provided a linear relationship is obtained) is the conversion factor needed to convert ETR into P^B and thus equals the efficiency electron yield (Φ_e) for O_2 evolution or C-fixation (mol O_2 produced or CO_2 fixed per mol electron produced. Thus:

$$P^B = \Phi_e \bullet ETR \qquad (7a)$$

Theoretically the optimal value for Φ_e is 0.25, which means that in the case of O_2-evolution 4 absorbed photons give rise to 4 charge separations and thus the production of 4 electrons. In the case of C-fixation we need to know the photosynthetic quotient (PQ) if we like to convert rates of O_2-evolution to rates of C-fixation. Thus:

$$P^B\ (\mu mol\ O_2\ (mg\ chl\textit{a})^{-1}\ s^{-1}) = \Phi_e \bullet ETR \qquad (7b)$$

$$P^B\ (\mu mol\ C\ (mg\ chl\textit{a})^{-1}\ s^{-1}) = \Phi_e \bullet ETR \bullet PQ^{-1} \quad (7c).$$

As explained above, two different methods to active fluorescence have been developed: the Pulse Amplitude Modulated (PAM) principle which uses a MT-flash to measure F_m and the Fast Repetition rate Fluorometry (FRRF)[2]. It should be noted that when the MT method is used (eq. 1, 3) with a fluorometer giving a ST-flash, the F_m ($F_{m\text{-}ST}$) measured is lower than that measured with a MT-flash ($F_{m\text{-}MT}$). As a consequence a lower PSII efficiency is obtained and thus a lower rate of ETR, because a ST flash leads to a single reduction of all Q_A, whereas an MT flash also reduces the Q_B and PQ-pool, leading to a higher F_m (Schreiber et al. 1995, Koblizek et al. 2001, Suggett et al. 2003, Kromkamp and Forster, 2003). ETR measured using a PAM fluorometer can be up to ca. 35% higher than ETR measured using a FRRF (Suggett et al. 2003, Kromkamp and Forster 2003).

At room temperature, most of the fluorescence emission originates from PSII and because charge separation and the splitting of water in the oxygen evolving complex both take place in RCII it might be expected that ETR and the rate of gross O_2-evolution show a tight coupling. As carbon fixation (the "dark reactions") takes place 'later' and is a 'more distant' step of the photosynthetic process, a more loose relationship might be obtained. Interestingly some diatoms seem also be able to use a C4-like C-fixation process using phosphoenolpyruvate carboxykinase (PEPCKase) or phosphoenolpyruvate carboxylase (PEPCase) (Cabello-Pasini et al. 2001, McGinn and Morel 2008) but whether MPB diatoms also possess this capacity has not been investigated yet.

Table 2 presents a compilation of studies where rates of ETR are compared to rates of photosynthesis obtained using more classical methods. Only a small number

[2] Several manufacturers make PAM-like fluorometers (e.g. Walz, PSI, Hansatech, Moldaenke). Fluorometers using the ST-protocol are made by Chelsea Instruments, Satlantic, PSI (and some Walz models). The former 2 use the FRRF protocol and can apply MT and ST flashes.

(12%) of these studies deal with biofilms. Most of these studies (85%) concern marine organisms, and *ca*. 70% relate to field studies or use natural samples photoacclimated in the laboratory for different periods of time. Natural phytoplankton studies mainly used the ST-approach; 5 studies used the 'pump and probe' and 18 used the FRRF techniques (mostly Chelsea's FAST^{tracka}), whereas only 6 studies used a PAM-type fluorometer in natural systems. The MT-PAM fluorometer is the apparent method of choice for seagrasses and macroalgal studies (16 out of 16) and both types are used for culture studies of unicellular algae. Fluorescence estimates of photosynthesis (from which ca. 20% are expressed as rETR, see 4 above) have been compared mainly with oxygen-evolution data (39 out of 66 references, including all the macroalgal/seagrass studies), and 4 dealing with $^{18}O_2$ and $^{17}O_2$). In 27 out of the 67 references cited in Table 2 fluorescence was compared to a ^{14}C-based method, the majority reporting on phytoplankton productivity. A limited number (6) compared ETR to both oxygen evolution and carbon fixation. 35% of the studies combined the different photosynthesis measurements simultaneously on the same sample, and 11 out of the 67 studies ran experiments in parallel (same time, but on different subsamples).

A global survey of Table 2 shows that in more than 75% of the studies cited, a good correlation was observed between rETR and other methods to estimate photosynthesis. Usually fluorescence methods gave higher estimates of photosynthesis, i.e., the value of Φ_e and/or PQ was overestimated (eq. 7). The PQ value depends on the N-source for growth and is close to 1 when ammonia is the sole nitrogen source and 1.3 when nitrate is the N-source for growth (Williams 1993). This is because the reduction of nitrate requires photosynthetically produced electrons. Many studies, however, show a large variation in PQ, which is at least partly due to

methodological errors (Williams and Robertson 1991). Another reason for the overestimate of C-fixation by ETR is that often an a priori assumption is made of Φ_e, which is assumed to equal 0.25 (= 1 mol oxygen per 4 mol photons absorbed). However, culture work shows that this optimal value is often not reached, and that it is closer to 6-7 mol oxygen per 4 mol photons absorbed (Flameling and Kromkamp 1998). For this reason Raateoja et al. (2004) used a value of 0.18 for Φ_e and assuming a PQ of 1.5, Kromkamp et al (in press) calculated that Φ_e equalled 0.15. Another reason for an overestimation might be that the chosen value for n_{PSII} when using the ST-method might be overestimated. This is discussed above in section 5 on rETR vs ETR.

MPB hardly features in the table (12%) and no direct estimates of C-fixation are made using a-priori assumptions for Φ_e. This is because the optical conditions in the MPB are so complex that absolute estimates of ETR are very difficult to make, related to the fact that quantification of rETR is hindered by difficulties in measuring the absorption coefficient of MPB, the presence of vertical migration in muddy sediments and the artefacts caused by deep layer fluorescence. These issues are discussed in the section on rETR vs ETR. To circumvent this problem several approaches have been tried: Barranguet and Kromkamp (2000) measured rETR at the sediment surface and compared the photosynthetic parameters obtained from those with those obtained from ^{14}C-fixation made on sediment slurries. Assuming no vertical migration and a homogenous chl a distribution with depth they calculated a conversion factor (called EE) and estimated depth integrated primary production. Their results demonstrated that with the assumptions mentioned, a single conversion factor could be used to estimate primary production throughout the year at different stations. At high light non-linearity was often observed, which could be due to

physiological factors (see below) but which could also be due to deep layer fluorescence, although that was not realized by the authors at the time. The EE factor calculated by Barranguet and Kromkamp (2000) corresponded with the conversion factor obtained from a culture study with the benthic diatom *Cylindrotheca closterium* (Morris and Kromkamp, 2003). Because the approach taken by Barranguet and Kromkamp (2000) did not take different vertical biomass distribution profiles in consideration. Serôdio (2003) developed a chlorophyll fluorescence based index to estimate photosynthesis:

$$P_{fluo} = E \frac{F_o}{F_{o,sed}} \Delta F / F_m' \quad (8a)$$

Basically this equation approximates to:-

$$P_{fluo} = F_o \bullet rETR \quad (8b)$$

where F_o in eq. 8a is corrected for background fluorescence of the sediment (and which should always be done as well as possible). The rationale of this approach is that F_o is a good proxy for the [chl a] in the photic zone of the sediment, and thus reflects the vertical biomass profile. The author compared the photosynthesis estimates of the fluorescence index with those obtained from microelectrode mechanisms (gross oxygen production using the light dark shift technique, Revsbech and Jørgensen, 1983) and found in general a linear relationship between both methods and the slopes did not differ significantly, suggesting that this index might be a powerful method to estimate MPB primary production. The validity of this model was corroborated using a model by Forster and Kromkamp (2004) (Fig. 7.1) who showed that the slope between the modelled and measured production depended little on the shape of the vertical biomass profile of the MPB. Recently Serôdio et al. (2007) confirmed the validity of the model results by demonstrating a linear relationship

between the fluorescence index shown in eq. 8a and measurements obtained using oxygen microelectrodes. As in the model study by Forster and Kromkamp (2004), and also demonstrated in a laboratory study with light and temperature treatments simulating seasons (Lefebvre et al. 2007), the slope of the regression between both methods varied throughout the year (Fig. 7.2), thus requiring a regular calibration of the conversion factor. If we consider only the studies for which the measurements were made simultaneously, in all but two studies (Flameling and Kromkamp, 1998; Hanelt and Nultsch, 1995), was the relation between fluorescence-based and oxygen-based estimates of photosynthesis linear. In most cases, ETR closely matched oxygen- or carbon-based estimates of photosynthesis.

On the other hand, in less than 25% of the references cited, a non linear, or a weak relation was observed. Usually, the non-linearity between P^B and ETR was observed at high irradiances above E_k . This is a general pattern, independent of the taxonomic level, as it can be observed in seagrasses, macroalgae, microalgae and in cyanobacteria. This non-linearity where ETR became relatively higher than P^B was attributed to light dependent O_2 consuming processes (photorespiration, chlororespiration, mitochondrial respiration in light, Mehler reaction (Flameling and Kromkamp 1998)) or other alternative electron sinks (e.g. thioredoxin, nitrogen reduction , Lomas and Glibert 1999), alternative electron pathways (cyclic transport around PSII and/or PSI, Lavaud et al. 2002), transfer of reductants from chloroplasts to mitochondria (OAA-malate and DHAP-PGA shuttles, Behrenfeld et al. 2004), different time-scale between fluorescence- and oxygen- or carbon-based measurements, methodological constraints in determination of the fraction of light absorbed by PSII (especially in biofilms, see section on rETR vs ETR), difference in apparatus sensitivity, vertical migration and contribution of subsurface cells in the

case of MPB. Moreover, non-linearity could be also light dependent, varying according to the light level experienced by the cells or to their light history, which in turn greatly influences the light absorption (or utilization) characteristics needed to estimate ETR.

Thus, in spite of an increasing number of studies dedicated to study the variation of photosynthetic parameters in microalgae using fluorometry and to compare them with other methods, many uncertainties still remain. This calls for a more in depth study of how the different parameters needed to calculate absolute ETR (a^*_{PSII}, n_{PSII}) and convert these to rates of C-fixation (Φ_e, PQ) vary and depend on nutrient status, light histories and growth rate (e.g., see discussion in Morris and Kromkamp (2003), and this applies especially to organisms producing biofilms, with their specific constraints.

8. General summary

The application of variable chlorophyll fluorescence to microphytobenthic (MPB) biofilms in soft sediment systems is complex as a result of the signal emanating form subsurface cells, cellular vertical migration within the sediment matrix, a high capacity for down regulation, chlororespiration in the dark and the effects of the physical structure of the sediment/biofilm matrix (light attenuation by the sediment matrix) itself. Despite these factors, fluorescence has yielded a large and valuable range of information on the ecology, physiology and productivity of MPB biofilms and indeed at the species level of diatoms, cyanobacteria and other taxa comprising them. Care in design of methods, including timing measurements away from periods of maximal migration and minimising measurement duration, as well as the use of numerical simulations to deconvolute depth-integrated measurements

(Serôdio 2004) have enabled correct interpretation of data, enhancing our knowledge of biofilm functioning and (photo)physiology. Fluorescence has provided valuable information on down regulatory measurements including xanthophyll cycling in the form of non photochemical quenching and PS II electron cycling (Perkins et al. 2006, Serôdio et al. 2005, 2006, Herlory et al. 2007, Lavaud et al. 2007, Cruz and Serôdio 2008). We now have a far better understanding of the use of fluorescence as a biomass proxy (Honeywill et al. 2002, Jesus et al. 2006), in the calculation of ETR and the construction of light response curves (Perkins et al. 2006, Serôdio et al. 2006, Herlory et al. 2007, Cruz and Serôdio 2008) and particularly encouraging is the correlation between fluorescence methodology and alternative methods such as oxymetry and carbon uptake (Table 1) for measurement of productivity (Barranguet and Kromkamp 2000, Forster and Kromkamp 2004, Kromkamp and Forster 2003, Morris and Kromkamp 2003, Morris et al. 2008, Serôdio et al. 2005, 2006, 2007). The application of fluorimetry to the MPB is still comparatively at an early stage and there is much more to be done to investigate the complex photophysiology of the species comprising the biofilms as well as in developing applied outputs of models of productivity and other ecosystem functions of these important ecosystems.

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Table 1.

Species	Growth conditions[1]	n_{PSII}	Source
Thalassiosira weisflogii	LL HL	723 552	(Dubinsky et al., 1986)
Isochrysis galbana	LL HL	636 264	(Dubinsky et al., 1986)
Prorocentrum micans	HL LL	725 513	(Dubinsky et al., 1986)
Dunaliella tertiolecta	LL HL	710 833	(Falkowski et al., 1981)
Skeletonema costatum	HL LL	590 605	(Falkowski et al., 1981)
Skeletonema costatum	LL-sinusoidal ML-sinusoidal	467 376	(Kromkamp and Limbeek, 1993)
Skeletonema costatum	LL-fluctuating ML-fluctuating	290 237	(Kromkamp and Limbeek, 1993)
Chlorella pyrenodiosa	LL HL	588 390	(Myers and Graham, 1971)
Emiliania huxleyi B11	LL HL	665 488	(Suggett et al., 2007)
Emiliania huxleyi B92	LL HL	720 528	(Suggett et al., 2007)

[1] LL= low light, ML = medium light and HL = high light growth conditions

Table 2 Comparison of photosynthetic parameters in aquatic organisms obtained with fluorescence-based methods and with carbon- and oxygen-based methods

Authors (year)	**Methods**		**Organisms**	**Growth irradiance / photoacclimation**	**Observed relationship**	**Hypothesis for NL**	**Remarks**
	Fluorescence	O_2 / ^{14}C			Linear*/Non Linear, Low-, Medium-, High-Irradiance		
Babin et al (1996)	FRRF	^{14}C	Phytoplankton Natural samples	Ambient conditions	NL	Nutrient limitations	
Barranguet and Kromkamp (2000)	PAM-101	^{14}C	MPB Natural samples	Ambient conditions	Mostly L, sometimes deviations at HI No significant correlation for P_m, E_k or □ between PAM and ^{14}C data	Migration, change in a* and light absorption by sediment	Measurements in parallel
Beer et al. (1998)	Diving-PAM (LC, RLC)	O_2	Seagrasses (*Zostera marina*, *Cymodocea nodosa*, *Halophila stipulacea*)	Ambient conditions	L (+, =, -) or NL (deviation at HI), depending on the species	Photorespiration and other O_2 consuming reactions	Simultaneous measurements (LC)
Beer and Björk (2000)	Diving-PAM	O_2	Seagrasses (*Halophila ovalis, Halodule wrightii*) Natural samples	Ambient conditions	L (+, =) or NL (deviation at HI), depending on the species	Photorespiration, estimation of light absorbed by PSII	Simultaneous measurements
Beer et al. (2000)	Diving-PAM, PAM-101	O_2	Macroalgae (*Ulva lactuca*, *U. fasciata*) Natural samples	*U. lactuca* : 1700 µmol m^{-2} s^{-1} *U. fasciata* : ambient conditions or 50 or 150 µmol m^{-2} s^{-1}	L (=)		Simultaneous measurements
Beer and Axelsson	PAM-101,	O_2	Macroalgae (*Ulva lactuca*,	Acclimation to 400	Mostly L	□$_{PSII}$ lower than 0.1	Simultaneous

(2004)	Diving-PAM		*Fucus serratus, Laminaria saccharina, Palmaria palmata, Porphyra umbilicalis*)	µmol m^{-2} s^{-1} *L. saccharina* : 100, 400 µmol m^{-2} s^{-1} *U. lactuca* : 200, 1700 µmol m^{-2} s^{-1}	NL at HI		measurements
Boyd et al (1997)	PPF	^{14}C	Phytoplankton assemblages (field), dominance of coccolithophorids	Ambient conditions	NL for □ L for E_k Partially L (-) for P_m	Limitations in the determination of some parameters (esp. bio-optical), differences in light sources and measurement duration	Significant correlations observed, but parameter values differed
Cabello-Pasini and Figueroa (2005)	Diving-PAM	O_2	*Ulva rigida*	Different DIC, irradiances and temperatures	Mostly L (=)	Lower correlation at HI and high nitrate concentration	Simultaneous measurements
Carr and Björk (2003)	Diving-PAM, PAM-2000 (LC, RLC)	O_2	*Ulva reticulata, U. fasciata*	50 µmol m^{-2} s^{-1}	NL, especially at HI or high DIC L with fresh sample at each light step P_{mf}/P_m changing with I	Light history, changes in □$_{PSII}$	Simultaneous measurements
Carr and Björk (2007)	PAM-2000	O_2	*Ulva fasciata*	80 µmol m^{-2} s^{-1} Nutrient limitation	Stable ratio (=) P_{mf}/P_m		Simultaneous measurements at one saturating non-inhibitory irradiance level
Corno et al (2005)	FRRF (FAST^{tracka})	^{14}C	Phytoplankton assemblages (field)	Ambient irradiance	L (+) Divergence in primary production estimates	O_2 consumption processes (mitochondrial, photorespiration, Mehler reaction), species composition, vertical distribution	
Estevez-Blanco et al (2006)	FRRF (FAST^{tracka})	^{14}C	Phytoplankton assemblages (field)	Ambient irradiance	L (+)		
Falkowski et al (1986a)	PPF	O_2	*Thalasiossira weissflogii,*		L at MI,	Quenching phenomenon	Measurements of

			Skeletonema costatum, Isochrysis galbana, Dunaliella tertiolecta, Chlorella vulgaris		deviations at LI and HI	(LI), cyclic transport of electrons around PSII (HI)	steady-state O_2 evolution
Falkowski et al (1986b)	PPF	O_2	*Chlorella pyrenoidosa*		L (=) when Φ_{PSII} >0.2 Deviation at HI	Cyclic transport of electrons around PSII	Simultaneous measurements
Falkowski et al (1991)	PPF	^{14}C	Phytoplankton Natural samples	Ambient conditions	L (mostly =)		
Flameling and Kromkamp (1998)	PAM-101	O_2	*Emiliana huxleyi, Scenedesmus protuberans, Phaeocystis globosa, Phaeodactylum tricornutum*		Mostly NL (deviation at HI) Sp. dependent	O_2 consumption processes or cyclic/pseudo cyclic transport of electrons,	Simultaneous measurements at steady state
Franklin and Badger (2001)	PAM-101	$^{18}O_2$ (mass spectro metry)	*Porphyra columbina, Ulva australis, Zonaria crenata* Natural samples	100 µmol m^{-2} s^{-1}	L (+) at LI NL at HI Sp. dependent	Quenching phenomena, cyclic transport of electrons around PSII, not photorespiration	Simultaneous measurements at steady state
Fujiki et al (2007)	FRRF (FAST*tracka*)	^{14}C	*Dunaliella tertiolecta*	Different light histories	L (+) at LI and MI NL at HI	Cyclic (PSII) or pseudo-cyclic transport of electrons	
Fujiki et al (2008)	FRRF (Diving Flash)	O_2	Phytoplankton Natural samples	Ambient conditions	Good correlation		FRRF- and O_2-data from different field campaigns
Geel et al (1997)	PAM-101, Xenon-PAM	O_2	*Phaeodactylum tricornutum, D. tertiolecta, Tetraselmis sp., Isochrysis sp., Rhodomonas sp.*	100 µmol m^{-2} s^{-1}	L (+) at LI NL above light saturation	Species dependent O2 consumption process (Mehler-type, not photorespiration)	Simultaneous measurements at steady state
Gilbert et al (2000a)	Xenon-PAM	^{14}C	Freshwater natural samples		Primary production overestimated by fluorescence	Duration of measurements package effect, Mehler reaction, N_2 reduction photorespiration	
Gilbert et al (2000b)	PAM-101	O_2	Freshwater species, *Chlorella vulgaris, Cryptomonas ovata, Cyclotella meneghiniana, Synechococcus leopoliensis*	30 µmol m^{-2} s^{-1}	L (=) below E_k / NL above E_K, depending on the species	Package effect, Mehler reaction, cyclic transport of electrons (PSII), not photorespiration	Simultaneous measurements
Glud et al (2002)	Diving-PAM	O_2, ^{14}C	Sea-ice algae		Mostly NL	Self-shading	RLC

Häder et al (1997)	PAM-2000	O_2	*Cladophora prolifera, C. pellucida* Natural samples	Ambient conditions	Good correlation		Follow-up of diel photoinhibition and recovery
Hancke et al (2008a)	PAM-101	O_2, ^{14}C	*Prorocentrum minimum, Prymnesium parvum, Phaeodactylum tricornutum*	Continuous light 80 µmol m^{-2} s^{-1}	Mostly L (+, =) □ , E_k, to a lesser extent P_m	Species-specific differences (especially for P_m) Mehler reaction	Measurements in parallel Fluorescence at steady state
Hancke et al (2008b)	PAM-101	O_2	*Prorocentrum minimum, Prymnesium parvum, Phaeodactylum tricornutum*	Continuous light 80 µmol m^{-2} s^{-1}	L (-, =, +), depending on the method High correlation for P_m, weak for □ and E_k	Differences in slope coefficient in function of the estimation of light absorbed by PSII	Simultaneous measurements at steady state Different approaches to estimate quanta absorbed by PSII
Hanelt and Nultsch (1995)	PAM-2000	O_2	*Palmaria palmata* *Natural samples*		NL	Differences in the role of pigments	Simultaneous measurements
Hanelt et al (1995)	PAM-101	O_2	*Dictyota dichotoma* *Natural samples*		L		Simultaneous measurements
Hartig et al (1998)	PAM-101	^{14}C	MPB (concentrated cell suspensions; aliquots for both methods)	Collection of motile fraction from natural sample	L (mostly -) at MI NL at LI, HI	Differences in light sources (LI), alternative electron sinks (Mehler reaction, nitrogen reduction) (HI)	Measurements in parallel. High correlation for Pm, not for □ and Ek. Measure of a* problematical
Heinze et al (1996)	PAM-101	O_2	*Scenedesmus obliquus* Freshwater species	100 µmol m^{-2} s^{-1}	L (=)		Simultaneous measurements at steady state
Kaiblinger and Dokulil (2006)	FRRF (FAST*tracka*)	^{14}C	Freshwater phytoplankton Dominant organisms : *Planktothrix rubescens* (summer) or diatoms		L (=) at subsaturating I	Influence of high surface irradiance	Vertical profiles
Koblizek et al (1999)	PAM-101, PEA, PSI	O_2	*Spongiochloris sp* Freshwater species.	20 µmol m^{-2} s^{-1}	Mostly L (=)		Exposure for 120 min to 1000 µmol m^{-}

							^{2} s^{-1}, recovery for 90 min in the dark
Kolber and Falkowski (1993)	PPF	^{14}C	Phytoplankton Natural samples		L (mostly =)		Measurements in parallel for fluorescence day profiles
Kromkamp et al (2001)	PAM-101	O_2	*Planktothrix rubescens* Natural freshwater samples		L (generally +) or curvilinear Deviation mostly at HI	Electron sinks (light respiration, Mehler reaction)	Simultaneous measurements Steady state ?
Kromkamp et al (2008)	Water-PAM and FRRF (FAST^{tracka})	^{14}C	*Lake phytoplankton*	Ambient conditions	L (+)		Both FRRF and PAM overestimate C-fixation
Kroon et al (1993)	PAM-101	O_2, ^{14}C	*Heterocapsa pygmeaea* Marine species	Low irradiances of different qualities, 9-19 µmol m^{-2} s^{-1}	Mostly L (=) Close coupling with O_2 data, weaker with ^{14}C	Changes in linear or cyclic electron transport rates, or enzymatic processes, depending on chromatic adaptation	Simultaneous measurements at steady state (O_2)
Kroon (1994)	PAM-101	O_2	*Chlorella pyrenoidosa* Freshwater species	Fluctuating growth irradiance, total daily dose 10.5 mol m^{-2} d^{-1}	L (-)		Simultaneous measurements at steady state
Kühl et al (2001)	Diving-PAM (LC, RLC)	O_2	Brown macroalgae, coralline red algae Natural samples		Good correspondence		
Lefebvre et al (2007)	FMS1	O_2	*Skeletonema costatum*	Simulated seasonal conditions (5 combinations of irradiances, photoperiods and temperatures)	L (+)	The slope of the relationship varied according to the seasonal treatment	Measurements in parallel, at steady state
Longstaff et al (2002)	Diving-PAM (ETR measured at diel ambient irradiance,	O_2	*Ulva lactuca*	Diel ambient irradiance Nutrient enrichment experiments	L at LI (=) and MI (+) depending on ETR determination	Cyclic transport of electrons around PSII, NPQ, Mehler reaction, photorespiration Accuracy of photon	Simultaneous measurements at steady state (diel ambient irradiance)

	and by RLC)				NL at HI	absorbance by thalli	
Masojidek et al (2001)	PAM-101	O_2	Freshwater cyanobacteria Natural samples	Diel ambient light	Mostly L (+) at LI and NL at HI Diel changes	Photorespiration, Mehler reaction	Simultaneous measurements possibly at steady state
Melrose et al (2006)	FRRF (FAST*tracka*)	^{14}C	Coastal phytoplankton	Ambient conditions	L (-)		FRRF mainly underestimated C-fixation
Migné et al (2007)	Diving-PAM	CO_2 (IRGA)	MPB Natural assemblages	Diel ambient light, screened by the Perspex dome for benthic chambers	Mostly L NL at HI, depending on season and station	Migration, relative contribution of surface/subsurface cells	Measurements in parallel on different samples
Moore et al (2003)	FRRF (FAST*tracka*)	^{14}C	Phytoplankton Natural assemblages	Ambient light regimes	L (+) between ^{14C} and FRRF-modelled productivity estimates NL (□)	O_2 consumption processes or non linear transports of electrons	Spectral differences between light source (underwater light field *in situ*, incubators)
Morris and Kromkamp (2003)	PAM-101	O_2	*Cylindrotheca closterium*	Two growth rates, 7 temperatures (short-term change)	Mostly L NL at LI and HI	Alternative electron sinks (HI), variation in respiration rates (LI)	ETR/O_2 relationship hardly influenced by temperature
Morris et al (2008)	MiniPAM, Water-PAM	^{14}C	MPB in tidal mesocosms Measurements made on biofilms (ETR) and suspensions (ETR, ^{14}C)	200 µmol m^{-2} s^{-1}	Good correlation (=) ETR/P_m for suspensions, weaker for biofilms	Changes with growth phase, diel rhythm, migration, absorption cross-section of MPB,	Measurements in parallel (suspensions)
Perkins et al (2001)	Diving-PAM	^{14}C	MPB Natural assemblages	Ambient irradiance (100 and 50%)	L at LI, MI NL at HI	Vertical migration and relative contribution of surface/subsurface cells	Measurements in parallel in sample cores
Perkins et al (2002)	Xenon-PAM, High resolution imaging	^{14}C	MPB Natural assemblages	Ambient irradiance 220 µmol m^{-2} s^{-1} (Imaging system)	NL	PSI contribution, vertical migration, relative contribution of surface/subsurface cells	Measurements in parallel on sample cores

	system						
Prasil et al (1996)	FRRF	O_2	*Chlorella pyrenoidosa*	50 µmol m^{-2} s^{-1}	NL at HI		Redox state of PQ, cyclic transport around PSII
Raateoja and Seppälä (2001)	FRRF (FAST*tracka*)	^{14}C, O_2	Freshwater species *Nannochloris sp.* Marine species	39 and 314 µmol m^{-2} s^{-1}	NL	Pre-set parameters to convert electron flows into primary productivity, nitrogen reduction	Influence of growth irradiance, diel cycle and nutrient uptake
Raateoja (2004)	FRRF (FAST*tracka*)	^{14}C	Phytoplankton Natural assemblages	Ambient irradiance	L at LI NL at HI	Methodological discrepancy (measurement periods)	Deviation from linearity at irradiance > 200 µmol m^{-2} s^{-1}
Raateoja et al (2004)	FRRF (FAST*tracka*)	^{14}C	Phytoplankton Natural assemblages	Ambient irradiance (primary production), fluorescent tubes (P/E curves)	NL	Differences in light sources, inadequacy of FRRF for near-surface measurements	
Rech (2004)	FMS1	O_2	*Amphora coffeaeformis, Haslea ostrearia, Entomoneis paludosa, Phaeodactylum tricornutum, Porphyridium cruentum,*	100 µmol m^{-2} s^{-1}	L at LI and MI NL at HI		Species-dependent relation
			Skeletonema costatum, H. ostrearia, Dunaliella tertiolecta, P. cruentum, Spirulina platensis	75 and 350 µmol m^{-2} s^{-1}	L at LI and MI NL at HI		Relation varying with the species, and the growth irradiance
Rees et al (1992)	PAM-101 ?	O_2	*Dunaliella sp.* Marine species	40 µmol m^{-2} s^{-1}	NL	Cyclic transport around PSI and PSII, Mehler reaction, etc.	
Rysgaard et al (2001)	Diving-PAM (RLC)	O_2, ^{14}C	Sea ice algae Natural communities	Ambient irradiance	L		Calibration ^{14}C/rETR (in situ RLCs, and parallel measurements on ice

							cores)
Sagert and Schubert (2000)	PAM-2000	O_2	*Palmaria palmata*	7 to 568 µmol m^{-2} s^{-1}	Significant correlation for P_m and E_K for lab measurements		Simultaneous measurements
Sarma et al (2005)	FRRF (FAST*tracka*)	O_2 (triple oxygen isotopes, light-dark bottle incubation)	Phytoplankton Natural assemblages	Ambient irradiance	Weak correlation between gross oxygen production estimated by ^{17} anomaly, light-dark bottle incubation, and FRRF	Differences in time resolution of in situ changes in irradiance	Calibration ^{14}C/FRRF Comparisons with seasons
Sarma et al (2006)	FRRF (FAST*tracka*)	O_2 (triple oxygen isotopes, light-dark bottle incubation)	Phytoplankton Natural assemblages	Ambient irradiance	Godd correlation (ca. =) between daily integrated production estimated by ^{17} anomaly, light-dark bottle incubation, and FRRF		Short-term variations (hourly/daily changes)
Schofield et al (1995)	PAM-101	^{14}C	Antarctic ice algae Natural communities	Ambient irradiance	Weak agreement between UV-B photoinhibition estimated by fluorescence and ^{14}C fixation	Correlation between ^{14}C- and fluorescence-based estimates changed with time of the day	Experiments on UV-B inhibition of carbon fixation rates Measurements in parallel
Schreiber et al (1995)	Xenon-PAM	O_2	*Ankistrodesmus braunii* Freshwater species	80 µmol m^{-2} s^{-1}	L (ca. =) at HI and MI NL at LI	Cyclic PSII transport rate	
Serôdio et al (1998)	PAM-101	O_2	*Chlorella vulgaris*	50 µmol m^{-2} s^{-1}	L, slope varying with species		

			Phaeodactylum tricornutum *Spirulina maxima*				
Serôdio (2003)	PAM-101	O_2	*MPB*	Ambient irradiance	L		Changes in biomass profile due to vertical migration
Serôdio et al (2007)	WATER-PAM	O_2	Natural sediment cores *MPB* *Natural sediment cores*	Ambient irradiance	L		Depth-integration of subsurface fluorescence
Silva et al (1998)	PAM-2000	O_2	*Gelidium sesquipedale*	Ambient irradiance	L		Good correlation whatever the depth
Smyth et al (2004)	FRRF (FAST*tracka*)	^{14}C (Daily primary production, LC)	Phytoplankton assemblages Natural communities	Ambient irradiance	NL (usually higher, P_m and E_k lower, when FRRF-estimated)	Integration with time and depth of instantaneous fluorescence data	
Suggett et al (2001)	FRRF	^{14}C, O_2	Phytoplankton assemblages Natural communities	Ambient irradiance	Good correlation (, P_m usually higher, E_k lower, when FRRF-estimated)	FRRF estimation of photosynthetic unit size, respiration, alternative electron sinks	
Suggett et al (2003)	Xenon-PAM, FRRF (Chelsea)	O_2 (flash yield)	*Chaetoceros muelleri, Prorocentrum minimum, Dunaliella tertiolecta, Emiliana huxleyi*	50 or 75 µmol m^{-2} s^{-1} (LI) 400 or 375 µmol m^{-2} s^{-1} (HI)	NL (PAM vs FRRF), differences being lower at HI	Differences between PAM and FRRF measurements explained by distinct approaches (single- vs multiple turn-over)	
			Prochlorococcus marinus	5-10 µmol m^{-2} s^{-1}			
	FRRF (Chelsea)	$^{18}O_2$ (mass spectrometry)	*D. tertiolecta, Thalasiossira weissflogii, Nannochloris atomus*		L (-, =) depending on the species		Simultaneous measurements

*When the relation is linear, ETR estimated photosynthesis can be equal (=), over- (+), or under-estimated in comparison with carbon- or oxygen-based method.

Figure Legends

Fig 2.1. Figure 4 from Serodio 2004 Joao – please write new legend

Figure 2.2. Quantum efficiency of individual cells of five benthic diatom species obtained using high resolution fluorescence imaging. By this method, only cells at the surface of the sediment were analysed, reducing any effect from sub-surface cells.

Figure 2.3. Cross sectioned sample from a stromatolite showing the surface mixed community and sub-surface layer of cyanobacteria (Reproduced courtesy of P. Reid)

Figure 2.4. Surface mixed eukaryotic microalgae and cyanobacteria mat on a stromatolite, note the fluffy three dimensional structure of the mat.

Figure 2.5. Light response curves for two stromatolite samples using a fluorimeter probe in the top-down position. The first sample (closed symbols) refers to a layer of cyanobacteria measured at 4 mm depth in the ooids matrix, data clearly show a large decrease in rETR post scraping away of the surface ooids. The second sample (open symbols) refers to a layer 1 mm below the stromatolite surface, again rETR decreased significantly post scraping.

Figure 3.1. NPQ versus irradiance (PPFD, Photosynthetic Photon Flux Density) in different species of diatoms : in black, planktonic species from the open ocean (*Thalassiosira oceanica*), coastal (*Skeletonema costatum*) and estuarine (*Phaeodactylum tricornutum*) habitats, in white three benthic species isolated from the

microphytobenthos (MPB) in Baie de Bourgneuf (Atlantic coast, France). Adapted from Lavaud et al. (2007) (planktonic species) and unpublished data (benthic species).

Figure 3.2. (A) Simplified scheme of the xanthophyll cycle (XC) regulation. Co-factor requirement for the enzymes is shown as well as the pH optimum. - ΔpH means that the DT epoxidase is inhibited by high ΔpH under high light (Goss et al., 2006). DD, diadinoxanthin; DT, diatoxanthin. Adapted from Lavaud (2007). (B) pH dependence of DD and VX de-epoxidation in isolated thylakoid membranes of the diatom *Phaeodactylum tricornutum* (open circles) and spinach (closed squares). De-epoxidation is calculated as [DT/(DD+DT)] for the diatom and as [(ZX+0.5AX)/(VX+AX+ZX)] for the plant. VX, violaxanthin; AX, antheraxanthin; ZX, zeaxanthin. Adapted from Jakob et al. (2001).

Figure 3.3. (A) Fluorescence induction kinetics in a *Navicula phyllepta* low light culture (25 µmol photons m^{-2} s^{-1}) under a 370 µmol photons m^{-2} s^{-1} actinic light; application of saturating pulses to measure F_m' are indicated by downward arrows after 10, 30, 60 s and when fluorescence reached 'steady-state' (140 s); NPQ = (F_m-F_m')/F_m'. (B) Rapid Light Response Curves (RLCs) of the same low light culture acclimated at 100 µmol photons m^{-2} s^{-1} for 1 h. (C) Maximum Electron Transport Rate (rETR max) parameter as extracted from the RLCs built on N. *phyllepta* low (100 µmol photons m^{-2} s^{-1} for 1h) and high (400 µmol photons m^{-2} s^{-1} for 1 h) light acclimated cultures. PPFD, Photosynthetic Photon Flux Density. Adapted from Perkins et al. (2006).

Figure 3.4. Legend: Rapid Light Response Curves (RLCs) made *in situ* on an intact microphytobenthos (MPB) community (after 15 min dark adaptation) in the Eden estuary (Scotland). Each light step lasted 3 min and the PS II efficiency ($\Delta F/F_m'$) was measured at 30 s intervals. Despite the fact that F and F_m' only stabilized near the end of the 3 min exposure at each light step, $\Delta F/F_m'$ stabilized already in less than 30 sec and rETR measurement was only disturbed for the highest intensities (over 800 µmol photons.m^{-2}.s^{-1}) whatever the length of the light steps (from 30 to 180 s). (Kromkamp J., unpublished).

Fig. 5.1. Comparison between $rETR_{max}$ (left Y-axis) and absolute ETR_{max} (right Y-axis, expressed per cell) for 4 different algal species: *C. autotrophica, T. pseudonana, E. huxleyi* and *Synechococcus* CCY9502. On day 7 (arrow) the cells were resuspended in phosphate-free medium and were thus starved for P (unpublished data from Kromkamp).

Figure 6.1. Selected HRFI fluorescence images (at *F*') used in the construction of light response curves Perkins et al. (2002). Image (a) was obtained near the start of the light curve, at 380 µmol m^{-2} s^{-1} PAR and image (b) at 1150 µmol m^{-2} s^{-1} PAR. Note the change in surface biofilm from a mixed diatom and euglenid community to just *Euglena* spp. in less than 8 minutes. Scale bar is 100 µm long

Figure 6.2. Rapid light curves for cultures of *N. phyllepta* exposed to 1 h light acclimation period of low (a: 25 µmol photons m^{-2} s^{-1}) or high (b: 400 µmol photons m^{-2} s^{-1}) light. Light response curves were run with light step durations of 10, 30 and 60 s and with either increasing (up). Note the increase in $rETR_{max}$, lack of saturation

or down regulation for light curves with incremental increases in light level, as opposed to curves with decreasing light levels where saturation and down regulation occurred. From Perkins et al. (2006).

Figure 6.3. Non-sequential Light Curves for cultures of *N. phyllepta* run with light step durations of 10, 30, 60 s and reaching steady state (SS). Note the increase in $rETR_{max}$ as a function of the duration of light step and the lack of saturation for steady state light curves. From Perkins et al. (2006).

Fig. 7.1. Relationship between modeled primary production (Y-axis) and a fluorescence index (X-axis, slightly modified after Serodio, 2003). For the calculations one set a single set of photosynthetic parameters was chosen and the results were due to differently shaped depth profiles and thus a corresponding different contribution of fluorescence originating from layers within the photic zone

Figure 7.2**.**